of photosynthetic rhizarians, and yet a genome and most of our molecular data come from one of these. There are relatively few parasites, but again they are better studied than their free-living sisters. We know more about the fossil record of dead foraminiferans than we do of the biology of living ones. This is really just a symptom of a larger problem — free-living heterotrophic protists are one of the most difficult kinds of life to study, and this is precisely what most rhizarians are. What makes these bugs tick, and how they affect their environment is a tricky business to resolve, but resolving it will surely provide the highlights for the next stage of rhizarian research.

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Generation of infectious virus particles from inducible transgenic genomes

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Arboviruses like dengue virus, yellow fever virus, and West Nile virus are enveloped particles spread by mosquitoes, infecting millions of humans per year, with neither effective vaccines, nor specific antiviral therapies [1,2]. Previous studies of infection and virus replication utilize either purified virus particles or deficient genomes that do not complete the viral life cycle [1,2]. Here we describe transgenic *Drosophila* strains expressing *trans*-complementing genomes (referred to as 'replicons') from the arbovirus Sindbis [2]. We use this binary system to produce, for the first time in any metazoan, infectious virus particles through self-assembly from transgenes. Such cell-type specific particle 'launching' could serve as an attractive alternative for the development of virus-based tools and the study of virus biology in specific tissues.

Arbovirus genomes are positive-stranded RNA molecules that are capped and polyadenylated, thereby resembling cellular mRNAs (Figure 1A). However, unlike most eukaryotic messages, the genomic RNAs of some arboviruses like Sindbis are bi-cistronic, containing two open reading frames (ORF1 and ORF2) [2]. We have generated transgenic Sindbis genomes stably inserted into the fly genome, under the control of an inducible promoter. After inducing host-cell transcription of virus ORF1, which encodes the viral RNA-dependent polymerase (RdRP, or 'replicase'), viral replication can be induced in *Drosophila* [3]. We sought to induce self-assembly of infectious virus particles *in vivo* by adding the viral structural proteins (which include the glycoproteins necessary for membrane fusion). Structural proteins are encoded by virus ORF2, and translated from a 'subgenomic RNA' [2] generated by the viral replicase (Figure S1A). Analogous 'launching' of infectious particles has previously been reported in yeast [4] and in plants [5], but never in any metazoan.

We first generated a transgenic replicon reporter (SinR-GFP), by replacing ORF2 with GFP, under the control of the viral replicase (translated from ORF1 of the same RNA). As previously reported, expression of such a replicon was universally low [3]. We therefore inhibited the cellular RNA interference (RNAi) pathway, which has a strong antiviral role in *Drosophila* [6,7], using protein B2, a dominant inhibitor of RNAi from flock house virus [8]. We observed high levels of GFP expression when SinR-GFP and B2 were coexpressed in such diverse tissues as gut, muscles, salivary glands, trachea, and photoreceptors (Figure 1B). Similarly high levels were obtained when RNAi was inhibited using *Dcr2* mutants (Figure S1B).

We next provided structural proteins by expressing a template for the 'subgenomic RNA' *in trans* from a second 'deficient' transgene lacking ORF1, which encodes the viral replicase (DH-BB; Figure 1C). We homogenized flies expressing the *trans*-complementing transgenes SinR-GFP, DH-BB and B2 under *GMR*-GAL4 control. This fly debris was then used to infect a monolayer of cultured baby hamster kidney (BHK) cells, in which Sindbis virus replicates efficiently (see Supplemental Information published with this article online). Robust expression of GFP was detected in 1.5–5.4% of total BHK cells (Figure 1D), indicating that these cells had been infected with Sindbis, and translated the self-replicating GFP-expressing replicon. As the absolute number of GFP-positive cells depended on fly rearing conditions and BHK cell passage number, we calculated an 'infection coefficient' (Inf.; see Supplemental Information). An Inf. >1 indicates an experimental condition that produced more infectious particles than the positive control, while values <1 correspond to reduced infectivity. Virtually identical results were observed when RNAi was inhibited using *Dcr2* mutants (Inf. = 0.94 ± 0.53; Figure S1G) and lower numbers were observed when different tissues were targeted (neurons, photoreceptors, glia,

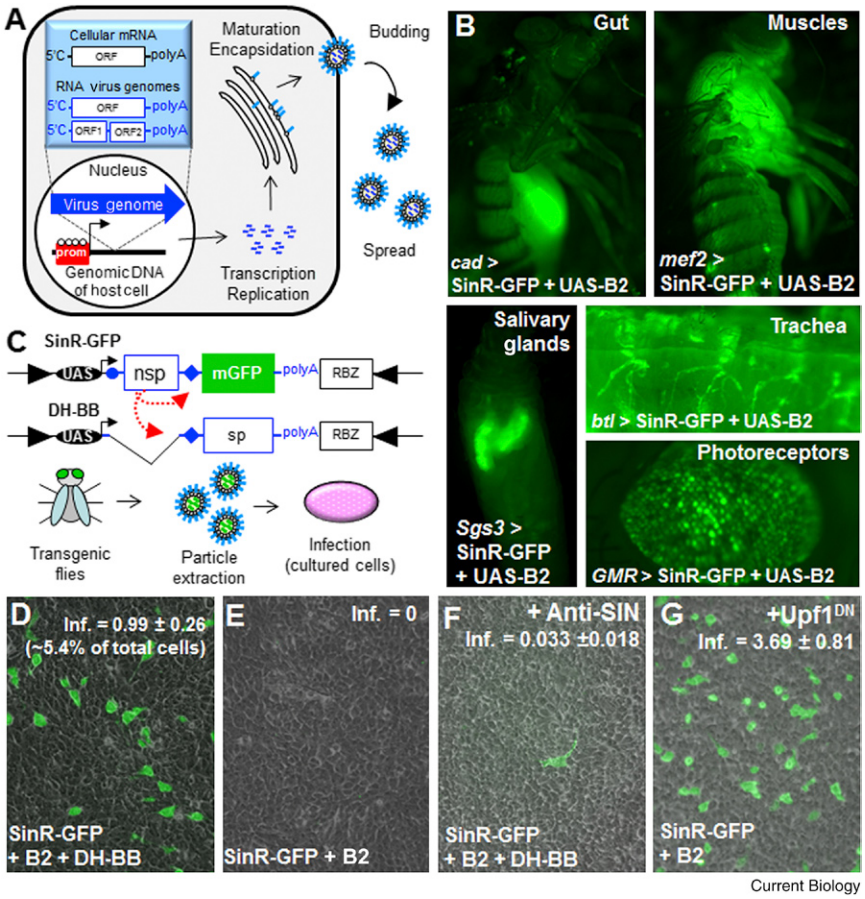

Figure 1. Generation of infectious Sindbis virus particles from inducible transgenes.
(A) Strategy for launching infectious virus particles *in vivo*, by inserting the single-stranded virus genome with (+) polarity into the host cell genome, under control of an inducible promoter (prom). Particles self-assemble from virus genomes transcribed by the host cell. Note the similarity to cellular mRNAs (cap, polyA). (B) Examples of viral RdRP-driven SinR-GFP expression *in vivo*, in combination with UAS-B2, in different tissues: gut (*cad*-GAL4), muscles (*mef2*-GAL4), salivary glands (*Sgs3*-GAL4), trachea (*btl*-GAL4), and photoreceptors (*GMR*-GAL4). (C) Schematic summarizing the strategy for self-assembly of infectious Sindbis particles. Structural proteins under control of the RdRP-dependent promoter (blue diamond) were provided *in trans* from a second, defective helper replicon in which the 'packaging signal' (blue circle) and ORF1 were deleted (DH-BB). Transgenic flies were homogenized and used to infect a monolayer of cultured baby hamster kidney (BHK) cells (see Supplemental Information). nsp, non-structural proteins; sp, structural proteins; RBZ, ribozyme. (D) Expression of replicon-derived GFP was observed in BHK cells infected with fly debris from animals expressing SinR-GFP + DH-BB + B2 under *GMR*-GAL4 control: ~5.4% of total BHK cells were positive for GFP. (Inf. = 'infection coefficient'; see text and Supplemental Information). (E) BHK cells never displayed GFP expression when homogenized flies lacked DH-BB, which provides structural proteins. (F) Antibody neutralization of the infectious fly debris using an antibody against Sindbis. Infectivity decreased by over 96% and recovered slowly upon dilution of the antibody (see Supplemental Information). (G) Inhibiting nonsense-mediated decay using UAS-Upf1^{DN} (see Supplemental Information) increased infectivity by a factor of ~3.7.

hemocytes; Figure S1H–L). Extracts from flies lacking the structural proteins (DH-BB) never resulted in GFP expression in BHK cells (Figure 1E). More importantly, 96% of the infectious potential was lost when infectious fly debris (SinR-GFP + DH-BB + B2) was incubated with a neutralizing antibody raised against Sindbis (Inf. = 0.033 ± 0.018; Figure 1F). Viral infection was gradually restored when the neutralizing antibody was diluted, and, importantly, a control antibody had no effect (Figure S2A–F). Thus, self-assembly of infectious virus particles can be genetically induced in a metazoan.

Finally, we examined whether an additional cellular pathway might inhibit viral infection. Bi-cistronic viral genomes often contain multiple stop codons [2], making them potential targets for mRNA quality control pathways, such as nonsense-mediated decay (NMD) [9]. We therefore generated a transgene that expressed a dominant-negative form of the crucial NMD effector Upf1 (Upf1^{DN}) [10]. Co-expression of UAS-Upf1^{DN} indeed led to a ~3.7-fold increase of GFP-positive BHK cells (Inf. = 3.69 ± 0.81) (Figures 1G, S2G–K). Thus, this assay provides an efficient way to identify pathways that affect viral particle formation.

Taken together, we present a new way of producing infectious virus particles in a cell-type-specific manner, making viral infections in metazoans independent of injections. This provides new approaches for tool development, and makes the study of viral infections amenable to dissection in genetic model organisms.

Supplemental Information

Supplemental Information includes two figures, construct sequences and experimental procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.12.009.

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